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Separation of Toxin and Hemagglutinin from Crystalline Toxin of *Clostridium botulinum* Type A by Anion Exchange Chromatography and Determination of Their Dimensions by Gel Filtration*

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SUMMARY

Crystalline preparations of *Clostridium botulinum* type A toxin were fractionated on DEAE-cellulose columns with Tris-HCl buffers at pH 8.0. The isolated toxic fraction was free of hemagglutinating activity and contained 5 times the specific activity of the crystalline toxin. The second fraction was a powerful hemagglutinin but was only feebly toxic; it emerged from the column as one or more peaks, under different elution conditions. By rechromatography and immunological tests, the toxicity of the second fraction was shown to be due to contamination with traces of the toxic fraction. By several criteria, the toxic and hemagglutinating components appeared to be at least two different proteins.

Most of the information obtainable from the ultracentrifugal analysis of these substances was also obtained by gel filtration on a Sephadex G-200 column and with much smaller protein concentrations. At physiological pH, the toxic fraction had a molecular weight of 150,000 and a Stokes radius of 48 A; these dimensions were in agreement with those established *in vivo* by other investigators. The hemagglutinin appeared to exist in three forms of aggregation with molecular weights of 290,000, 500,000, and 900,000.

Since the toxin of *Clostridium botulinum* type A was obtained in the crystalline form (1), its homogeneity has not been seriously questioned. However, observations (2, 3) showed that the crystalline toxin was not only highly toxic but also hemagglutinating, and that the hemagglutinin could be dissociated from the toxin without loss of toxicity (4) and with a two- to three-fold increase in specific activity (5). In addition, in the ultracentrifuge under certain conditions, the toxin appeared to contain at least two distinct components with significantly different $s_{20,w}$ values (5–7). This evidence did not change the notion that the crystalline toxin is a homogenous protein of molecular weight 900,000, since the multiplicity of components and the two different biological activities were ascribed to the properties of the same molecule in different states of aggregation (5).

Schantz, Stefanye, and Spero (8) attempted to fractionate crystalline toxin on a DEAE-cellulose column but obtained material with no greater toxicity than was observed in the crystalline toxin. Gerwing, Dolman, and Bains (9) have reported isolation of type A toxin of molecular weight 12,000 obtained from crude culture filtrates by 50% $(NH_4)_2SO_4$ saturation with subsequent purification of the isolated material on a DEAE-cellulose column at pH 5.6. Neither of these studies discussed the possible presence of the hemagglutinating activity in their purified material.

Observations made in our laboratory (10, 11) raised strong doubts as to the homogeneity of crystalline toxin. In a preliminary report (12), we demonstrated that crystalline toxin can be chromatographically resolved into two fractions, α and β, which exhibit different physical, chemical, and serological properties.

This communication describes the resolution of the crystalline toxin into at least two different proteins, a toxin and a hemagglutinin. The latter, in turn, is separable into three components of different molecular dimensions. Also reported is the estimation of the molecular dimensions of the isolated fractions of the crystalline toxin, particularly the toxic moiety α at low protein concentration and at approximately physiological pH. Finally we consider whether the α fraction represents the molecules that appear at the myoneural junctions where the toxin acts.

MATERIALS AND METHODS

Crystalline preparations of *C. botulinum* type A toxin were obtained through the generosity of Dr. E. J. Schantz, Fort Detrick, Frederick, Maryland. The crystals, stored in 0.9 M

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$(NH_4)_2SO_4$, were collected by centrifugation and dissolved in an appropriate buffer at room temperature. The dissolved toxin was freed from $(NH_4)_2SO_4$ by dialysis for 16 hours at 4° against 100 times the volume of the buffer to be used later in chromatography. Any residual turbidity was removed by centrifugation. The protein concentration and the index of purity of the toxin samples were determined as suggested by Schantz (13).

The sources of the other proteins used in this study were as follows: cytochrome *c* (horse heart, Sigma Type III, lot No. 1148–7150); bovine serum albumin (College of American Pathologists, Chicago, Ill., lot No. 18); glyceraldehyde-3-phosphate dehydrogenase (Calbiochem, lot No. 501536); aldolase (rabbit muscle, Calbiochem, lot No. 54588); catalase (Worthington, CTR 5665); β-galactosidase (14) (Dr. E. J. Steers, Jr., National Institutes of Health, Bethesda, Maryland). Blue dextran and Sephadex G-200 were obtained from Pharmacia Chemical Company Piscataway, New Jersey.

Three different batches of DEAE-cellulose (Cellex-D, Bio-Rad Laboratories) of exchange capacity 0.61, 0.70, and 0.78 meq per g were combined for use. The material was soaked in 1.0 N NaOH for about 2 hours, washed repeatedly with water to the pH of water, suspended in 1% HCl for an hour, and washed again with water to the pH of water. This cycle was repeated until the DEAE-cellulose changed from orange-yellow to white. After the last wash cycle, the cellulose was washed once with a buffer and suspended in the same buffer until ready for use. Columns of 0.9 cm were packed to a height of 32 to 34 cm with the DEAE-cellulose and equilibrated with about 500 ml of the desired buffer for about 16 hour. The bed height was adjusted to 30 cm (column bed volume ~ 28 ml), and glass wool was layered on top. All ion exchange chromatography was performed at 24 ± 2°. The columns were eluted at atmospheric pressure at flow rates of 25 to 35 ml per hour. The eluate was collected in 2.8-ml fractions with a Gilson fraction collector.

Preparation of Sephadex gel and packing and operation of the columns were carried out essentially following Andrews' method (15). Protein samples in 1 to 2 ml were layered over a thin glass wool layer covering the gel bed. Column flow rate did not exceed 30 ml per hour. The columns were operated at 24 ± 2°.

Buffers were prepared by titrating the acidic and basic conjugates of the same molarity to the desired pH; *e.g.* 0.067 M citrate-phosphate buffer, pH 5.6, was prepared by titrating 0.067 M Na_2HPO_4 with 0.067 M citric acid to pH 5.6. Phosphate buffer was prepared with Na_2HPO_4 and NaH_2PO_4 solutions. The concentration of Cl^- in Tris-HCl buffer was determined by titrating Cl^- against standard $AgNO_3$ solution with K_2CrO_4 as indicator. A calculated amount of Cl^- as NaCl was added to Tris-HCl buffer for linear and stepwise gradient elution.

For long Sephadex columns, 0.05 M Na_2CO_3-$NaHCO_3$ buffer, pH 9.2, was adjusted to 0.5% NaCl with solid NaCl. This caused a slight drop in pH, which was readjusted to pH 9.2 with Na_2CO_3 solution. Other buffers of pH 7.2, 7.5, and 8.0 were prepared by titrating 0.05 M solutions of Tris and HCl, both of which contained 0.1 M HCl.

The Stokes radius of the toxin molecules were determined by use of the equation

$$K_D = \frac{V_e - V_0}{V_i} = \left(1 - \frac{a}{r}\right)^2 \left[1 - 2.104\left(\frac{a}{r}\right) + 2.09\left(\frac{a}{r}\right)^2 - 0.95\left(\frac{a}{r}\right)^3\right] \quad (1)$$

describing the molecular sieve action of a Sephadex G-200 column (16, 17). K_D is the distribution coefficient of the eluted material, V_e is the elution volume of that material, V_0 is the void volume, and V_i is the effective internal volume of the column. V_0 and V_i were established according to Rogers, Hellerman, and Thompson (18) and Habeeb (19). The Stokes radius of the eluted molecule is denoted by a; the effective pore radius of the gel, by r.

The volume of the eluate collected from the Sephadex column (2.5 × 50 cm) in every fourth tube was measured, and the mean effluent volume per tube was calculated. The variation in volume from tube to tube was never more than ±2.5% from the mean volume of 2.5 ml. The V_e of a solute was estimated from the elution diagram by extrapolating the sides of the solute peak to the apex.

Protein concentrations of the fractions were determined spectrophotometrically in a Zeiss PMQ II spectrophotometer at 278 mμ and by measuring the fluorescent intensity in the Aminco-Keirs spectrophotofluorometer at 285 mμ excitation and 350 mμ fluorescent wave length. Nitrogen determination was carried out by a modification of Nessler's method (20). The nitrogen content of this protein was tentatively taken as 16.25%. In cases of low amounts of protein, where Beer's law at 278 mμ failed, an excellent linear relationship) was found between protein concentration and fluorescent intensity. This relationship held to one-fourth the limit of protein concentration measurable by absorbance and could be extended further by increasing the sensitivity of the apparatus. Thus, protein concentrations as low as 4 μg per ml could be measured. Amounts of protein eluted as individual components were estimated by adding fluorescent intensity and the volume of each fraction.

For rechromatography experiments, effluent containing the peaks from the first chromatographic run were dialyzed for 4 or more hours, with hourly changes, against a 10-fold volume of the buffer to be used. Linear gradients were generated by allowing 130 ml of buffer with high salt concentration to mix with 130 ml of the buffer used for equilibrating the column. Both vessels were of the same dimensions and were placed at the same horizontal level.

The toxicity was assayed for the number of minimal lethal doses by injecting 0.1 ml of the eluate intravenously into white mice weighing 20 g and noting their survival time (21). The hemagglutinating activity of the eluted samples was titrated by two methods. In a rapid test, a drop of human red blood cells in suspension was mixed with a drop of test material on a microscopic slide, and the reaction was noted with or without the aid of a microscope. Semiquantitative estimation of the potency of the hemagglutinins in the test solution was provided by adding 0.5 ml of a 2.0% suspension of human red blood cells in 0.9% NaCl solution to 0.5 ml of serially diluted eluates. The mixtures were incubated at 37° for 60 min and at 4° overnight. The most dilute test sample which caused cell clumping was recorded.

Ion Exchange Chromatography of Crystalline Toxin of C. botulinum Type A—In preliminary experiments, the toxin in 0.067 M citrate-phosphate buffer, pH 5.6, was applied on DEAE-Sephadex or DEAE-cellulose columns (0.9 × 30 cm) previously equilibrated with the same buffer. Immediately after the elution of 1 column volume, a sharp peak emerged. Further elution with the same buffer or with buffer of increased molarity yielded no more protein. The first indication of separation of crystalline toxin was noted when 0.05 M phosphate buffer, pH 6, was

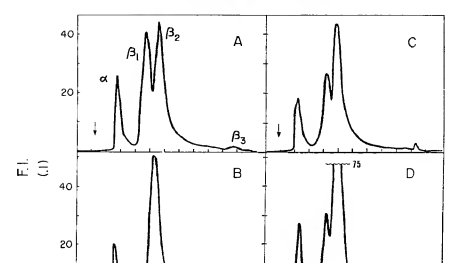

FIG. 1. *A* through *D*, chromatography of four different batches of *C. botulinum* type A toxin isolated and crystallized at four different times between a period of ½ to 2 years. DEAE-cellulose columns, 1 × 30 cm, were equilibrated with 0.15 M Tris-HCl buffer, pH 8. After 4.5 mg of toxin were applied, columns were eluted with the starting buffer until linear gradient elutions were started at the point indicated by *arrow*. Fraction size, 2.8 ml per tube; 0.1 is the instrument setting for fluorescence intensity (*F.I.*) measurement. The linear gradient was generated by allowing 130 ml of 0.15 M Tris-HCl buffer, pH 8, containing 0.5 M Cl^-, to flow into a mixing chamber containing 130 ml of the starting buffer. Elution was complete with 260 ml of salt gradient.

used with a linear NaCl gradient for elution. Clear separation of crystalline toxin into two components was achieved at a higher pH (12). The use of DEAE-Sephadex became impracticable because of significant shrinkage of the column bed in the presence of a salt gradient; therefore, DEAE-cellulose was used subsequently.

Toxin, 4.5 mg was applied on a DEAE-cellulose column, 0.9 × 30 cm, equilibrated with 0.15 M Tris-HCl buffer at pH 8.0. The column was washed with 2 column volumes of buffer, and a linear gradient was generated with 130 ml of buffer containing 0.5 M Cl^-. With increasing Cl^- concentration, four clearly distinguishable peaks emerged in the eluate (Fig. 1). These peaks were designated as α, β_1, β_2, and β_3, respectively. The α peak contained 78 to 80% of the toxic activity (minimal lethal doses) of the stock solution and had about 5 times its specific toxicity (~1.86×10^8 LD_{50} compared to ~3.87×10^7 LD_{50} per mg of protein). The β fractions strongly agglutinated red blood cells and were very feebly toxic.

Other batches of toxin, isolated and crystallized from culture filtrates of *C. botulinum* type A grown at different times (ranging from ½ to 2 years apart), were subjected to similar chromatographic procedures. Seven such batches were examined, and all resulted in qualitatively identical profiles, which differed only in the relative concentration of the four components. Fig. 1, *A* through *D* shows the chromatographic analysis of four of the seven samples. Crystallization of *C. botulinum* type A invariably resulted in material containing the same four characteristic components.

To establish the identity of the first peak (α) emerging from the DEAE-cellulose, the first half of the eluate, containing the α peak from DEAE-cellulose, was rechromatographed on DEAE-Sephadex. A single peak emerged at the same elution volume and Cl^- concentration as was obtained originally on DEAE-Sephadex (12). When the procedure was reversed, the α peak obtained from DEAE-Sephadex, when rechromatographed on DEAE-cellulose, also resulted in a single peak.

A clearer separation of the individual components of crystalline toxin was attempted by applying a stepwise Cl^- gradient. The α, β_1, β_2, and β_3 fractions emerged at 0.12, 0.19, 0.24, and 0.5 M Cl^-, respectively, in the buffer. NaCl was added to portions of 0.15 M Tris-HCl buffer, pH 8, to bring the net Cl^- concentration to the desired levels. These salt-enriched buffers were used, in order of increased salt concentrations, to elute the fractions of 6.4 mg of toxin applied on a DEAE-cellulose column, 0.9 × 30 cm. With each of the changes in Cl^- concentration, a peak corresponding to the α, β_1, β_2, β_3 fractions emerged (Fig. 2*A*). The relative concentrations of protein in these components were about 15, 13, 54, and 19%, respectively.

After readjustment to the ionic strength of the starting buffer, the four fractions were rechromatographed with a stepwise gradient as described above. The α peak emerged at the same elution volume as in the original run. Because of small initial yield of the β_1 and β_3 fractions, the respective fractions from two separate runs were pooled for rechromatography. The results obtained with the β_1, β_2, and β_3 fractions are shown in Fig. 2, *B* through *D*, respectively. The β_1 fraction resolved into four components emerging at the same Cl^- concentrations as were observed with the original toxin, in relative proportions of α = 5%, β_1 = 69%, β_2 = 23%, and β_3 = 3%. The β_2 fraction resolved similarly into four components in positions of α, β_1, β_2, and β_3. The relative proportions of these fractions were: α = 4%, β_1 = 14%, β_2 = 52%, and β_3 = 29%. The elution profile of β_3 showed traces of α and β_1, while β_2 and β_3 were found in concentrations of 69 and 31%, respectively. On rechroma-

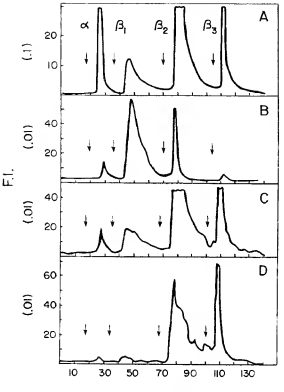

FIG. 2. Separation of the different components of crystalline toxin by stepwise gradient elution and their rechromatography on columns of DEAE-cellulose, 1 × 30 cm. The columns were equilibrated with 0.15 M Tris-HCl buffer, pH 8.0. Buffers containing 0.12, 0.19, 0.24, and 0.5 M Cl^- were applied in the increasing order at the positions marked with *arrow*. Fraction size was 2.8 ml per tube. Instrument sensitivities for recording fluorescence intensity (*F.I.*) in Fig. 2, *B* through *D*, were 10 times more than in Fig. 2.4. *A*, column loaded with 6.4 mg of toxin was washed with starting buffer until stepwise gradient elution was started. *B*, elution profile obtained when β_1, isolated from crystalline toxin (see Fig. 2.4), was rechromatographed with conditions as in Fig. 2.4. *C*, elution profile obtained when β_2, isolated from crystalline toxin (see Fig. 2.4), was rechromatographed with conditions as in Fig. 2.4. *D*, elution profile obtained when β_3, isolated from crystalline toxin (see Fig. 2.4), was rechromatographed with conditions as in Fig. 2.4.

tography, each of the β fractions yielded some α component. Contamination of the β peaks with the α fraction decreased with the distance of these peaks from the position of α.

In all experiments the α fraction remained highly toxic and free from hemagglutinins, whereas the β fractions were strongly hemagglutinating and still possessed slight toxicity. To determine the nature of toxicity of the β fractions, mice received intravenous injections of each of the three components. These were followed immediately by intraperitoneal injections of rabbit antiserum prepared against the α fraction. All mice treated in this manner survived. Corresponding controls without the anti-α serum died. This was considered as proof that the toxicity of the β fraction was due to the presence of the α fraction.

Sephadex Column Chromatography of C. botulinum Toxin—Sephadex G-200 columns can be used for the study of dissociation-association (22, 23) and for the determination of molecular weight (15) and Stokes radii of proteins at very low concentrations (16). The crystalline toxin and its isolated components were therefore examined by this method. About 7.69 mg of crystalline toxin in 3.0 ml of carbonate buffer, pH 9.2, placed on a Sephadex G-200 column (2 × 114 cm), equilibrated and eluted with this buffer, emerged in two peaks (Fig. 3). The peak emerging first contained 80% of the applied protein and was only feebly toxic, but strongly hemagglutinated human red blood cells. The second peak was slower in eluting, constituted 20% of the protein, and contained most of the toxin. Whereas the specific activity of the starting material contained 7.2×10^5 m.l.d./1.0 absorbance unit, the fast peak contained 4.8×10^2 m.l.d./1.0 absorbance unit and the slow second peak 7.0×10^6 m.l.d./1.0 absorbance unit.

Determination of Molecular Dimensions of Components of Crystalline Toxin—The α, β_1, β_2, and β_3 fractions were isolated from crystalline toxin with a DEAE-cellulose column with the use of a stepwise gradient. Aliquots (2.0 ml) of each were chromatographed on a Sephadex G-200 column, 2.5 × 50 cm, equilibrated and eluted with 0.05 M Tris-HCl buffer, pH 8.0, containing 0.1 M KCl (Fig. 4). The α fraction emerged as a single peak with no visible inflection points on the leading or trailing edge. The effluent following this peak contained no more fluorescent material. Aliquots taken from various tubes under the peak proved highly toxic. The concentration of protein at the apex was 20 μg per ml. The elution profile of the β_1 fraction showed the existence of at least two major components at V_e = 102.5 ml and 117.1 ml, and an inflection area at V_e = 129.3 ml. The β_2 fraction eluted as a single peak with V_e = 103.3 ml showing no inflection points on the curve. The elution profile of the β_3 fraction had a major peak at V_e = 105.4 ml, a shoulder at V_e = 87.4 ml on the leading edge, and an irregular trailing edge.

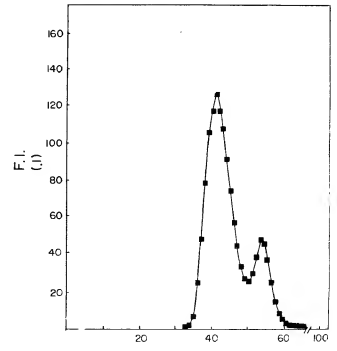

FIG. 3. Gel filtration of crystalline toxin, 7.69 mg in 3.0 ml, on a column of Sephadex G-200 (2 × 114 cm) with 0.05 M carbonate buffer, pH 9.2, containing 0.5 NaCl. Fraction size, 2.5 ml per tube. *F.I.*, fluorescence intensity.

A group of substances of known molecular weight (cytochrome c, bovine serum albumin, glyceraldehyde-3-phosphate dehydrogenase, catalase, β-galactosidase, and blue dextran) were eluted through the same column under the same conditions.

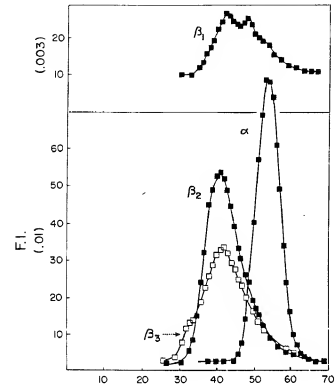

FIG. 4. Gel filtration of α, β_1, β_2, and β_3 components, isolated from crystalline toxin by anion exchange chromatography, on a column of Sephadex G-200 (2.5 × 50 cm) with 0.05 M Tris-HCl buffer, pH 8.0, containing 0.1 M KCl. In the upper part of the figure the elution profile of β_1 was recorded with higher instrument sensitivity than the other three in the lower part. Concentration of α in peak tube was 20 μg per ml. Similar elution profiles of α were obtained with buffers at pH 7.5 and 7.2 and also with different protein concentrations, so that the peak tube contained 13 or 52 μg of protein per ml. Fraction size, 2.5 ml per tube.

Each protein eluted as a single peak, with no inflection points; bovine serum albumin dissociated as expected into monomers and dimers. The V_e values of these substances (Tables I and II) plotted against the logarithms of their molecular weights yielded a straight line (Fig. 5). The molecular weights of the α, β_1, β_2, and β_3 fractions established from this curve, are shown in Tables I and II and Fig. 5.

The flow rate from the gel filtration column equilibrated with pH 7.2 buffer was too slow to elute all the test proteins. The molecular weight of the α fraction at this pH was therefore not obtained from a calibration curve. Instead, only blue dextran, aldolase, and the α fraction were eluted. Since the K_D value of the α fraction at this pH was similar to that at pH 8, and since the V_e values of aldolase and the α fraction were essentially the same, the molecular weight of the α fraction at pH 7.2 was taken as 150,000. At pH 8.0, with a total gel bed volume of 248 ml, the V_e values for the α component and blue dextran were 133.7 ml and 69.1 ml, respectively. These values for V_e, V_0, and V_t, substituted in Equation 1, yielded $K_D = 0.360$ for the α fraction. From a similar experiment with pH 7.5 buffer, the K_D of the α fraction was 0.355 ($V_e = 140.6$ ml, $V_0 = 85.8$ ml, gel bed volume = 248 ml), which was similar to the K_D obtained at pH 8.0 (mean deviation ± 0.7%).

TABLE II

Molecular weight, Stokes radii, and elution volume of toxic and hemagglutinin fractions isolated from crystalline toxin Clostridium botulinum type A

Crystalline toxin components	Experimentally derived		
	Molecular weight	Stokes' radii	V_e
	× 10^5	$m\mu$	ml
α	1.5	4.79	133.7
β_1	2.9	5.94	117.1
	5.2	7.19	102.5
β_2	5.0	7.13	103.3
β_3	4.6	6.96	105.4
	9.3	9.14	87.4

TABLE I

Molecular weight, Stokes' radii, and elution volume of different proteins and calibration of pore radius of Sephadex G-200 column

Proteins	From literature		Experimentally derived	
	Molecular weight[a]	Stokes' radii	V_e	Pore radius[b]
		$m\mu$	ml	$m\mu$
Cytochrome c	1.24 × 10^4	1.74[c]	197.6	22.8
Bovine serum albumin monomer	6.5–7.0 × 10^4		152.5	
Bovine serum albumin dimer	1.3–1.4 × 10^5		132.8	
Glyceraldehyde 3-phosphate dehydrogenase	1.15–1.45 × 10^5	4.13[d]	142.5	22.2
Aldolase	1.4–1.5 × 10^5	5.0[d]	133.5	23.8
Catalase	2.3–2.5 × 10^5	5.22[c]	125.6	22.6
β-Galactosidase	5.4 × 10^5		100.8	
Blue dextran	20 × 10^5		69.1	

[a] Taken from Andrews (15). Value for β-galactosidase from Craven, Steers, and Anfinsen (14).
[b] Mean effective pore radius is 22.85 with a mean deviation of ±2.50%.
[c] From Ackers (16).
[d] From Rogers *et al.* (18).

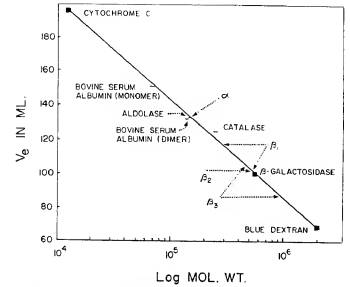

FIG. 5. Molecular weights of proteins as determined by gel filtration on Sephadex G-200 column (2.5 × 50 cm) with 0.05 M Tris-HCl buffer, pH 8.0, containing 0.1 M KCl. V_e is the elution volume of test substances plotted against their log (molecular weight). The molecular weights of the proteins are: cytochrome *c*, 1.24×10^4; bovine serum albumin monomer, 6.5 to 7.0×10^4; bovine serum albumin dimer, 1.3 to 1.4×10^5; aldolase, 1.4 to 1.5×10^5; catalase, 2.3 to 2.5×10^5; β-galactosidase, 5.4×10^5; and blue dextran, $\sim 2.0 \times 10^6$. Since β_1 and β_2 each showed presence of two components, the molecular weights of these were determined.

Gel filtration of the β_1 fraction revealed that the two components of β_1 had molecular weights of 2.9×10^5 and 5.2×10^5, respectively. Similarly, the β_2 fraction had two components with respective molecular weights of 4.6×10^5 and 9.3×10^5. The molecular weight of the β_2 fraction was 5.0×10^5. Thus, it appeared that the β_1 and β_2 fractions each contained a component with molecular weight very near that of β_2 (deviations of +4% and −8% from 5.0×10^5), such that β_2 appeared to be a common component of the other two β fractions.

The gel pore radii of the Sephadex G-200 column were established from known Stokes radii and experimentally determined K_D values of cytochrome *c*, glyceraldehyde-3-phosphate dehydrogenase, aldolase, and catalase. The mean effective pore radius of the gel was 22.85 mμ ± 2.5% (Tables I and II). From Equation 1 the value for the Stokes radius *a* for the α component was 4.79 mμ or 48 A.

DISCUSSION

The crystalline toxin of *C. botulinum* type A, which for the past 20 years had been considered to be in a high state of purity, was found on chromatographic analysis to contain at least four components. The α fraction proved to be the neurotoxin elaborated by these organisms. The three other components, slightly toxic but containing the hemagglutinating property of the crystalline toxin, were designated as fractions β_1, β_2, and β_3. Since the toxicity of the β fractions could be neutralized *in vivo* with rabbit antiserum prepared against pure α fraction, it was concluded that the toxicity of the β component was due to contamination with α. This was shown by rechromatography of the β components.

Our attempts to purify crystalline toxin with the use of the chromatographic conditions of Gerwing *et al.* (9) neither separated the hemagglutinins from the toxin nor increased the specific activity of the eluted material. Only at or above pH 6.0 in the presence of a Cl^- gradient did the resolution of the crystalline toxin become apparent. The failure to observe separation of crystalline toxin at pH 6.5 by Schantz *et al.* (8) may be due to the shortness of their column (10 cm). At pH 7.2, pronounced separation of the α and β fractions was achieved (12), although at this pH the α fraction was not completely free of hemagglutinins. Better separation of the α and β fractions was obtained at pH 8.0, at which point the α fraction became free of detectable hemagglutinating activity, and β resolved into three distinct fractions.

Gel filtration was used to examine the homogeneity of the isolated four components and to establish their molecular dimensions. Efforts to dissociate the toxin on a Sephadex G-200 column, 2 × 114 cm, at pH 9.2 resulted in two components of different molecular weights and different biological activities. Use of longer columns, up to 130 cm, did not improve the resolution. The reason for the incomplete separation of crystalline toxin (see Fig. 3) became evident when overlapping of elution profiles of different fractions was observed (Fig. 4). Occasionally, poorer resolution of the two peaks was noted with different batches of Sephadex. When this occurred, a single peak appeared with a skewed trailing edge. Aliquots from the last half of the trailing edge exhibited higher specific toxicity than the rest of the peak. Andrews (15) explained occasional poor separation of β-galactosidase from blue dextran as being due to differences in the degree of cross linking and, hence, to different water-regaining capacities of different batches of gels.

The symmetry in the elution profile of the α fraction, shown in Fig. 4, remained characteristic during gel filtration at various pH values and protein concentrations (the peak tube contained 13, 20, and 52 μg per ml of this protein). The K_D value for the α fraction also remained identical, which suggests that its molecular dimension remained essentially the same. This was in accord with the observations made in the ultracentrifuge (24); under all conditions tested $s_{20,w}$ of the α fraction was 7.25 ± 0.05.

Although there is no general agreement as to which dimensional parameter of a protein molecule most closely correlates with the gel filtration elution volume, it has been well established by Andrews (15), Whitaker (25), and Leach and O'Shea (26) that the molecular weight may be calculated from elution volume. Andrews (15) showed an excellent linearity between the elution volume and the log of molecular weight of some "well behaved" proteins. According to Ackers (16), Laurant and Killander (27), and Siegal and Monty (28), Sephadex G-200 columns can be used to derive molecular size of a protein molecule. By Andrews' method (15) the molecular weight of the α fraction was found to be 150,000 at pH 7.2, 7.5, and 8.0. By ultracentrifugal analysis the molecular weight of the α fraction, at pH 9.5, was found to be 128,000 ± 10% (24). In the ultracentrifuge Wagman (7) found a toxic moiety from the crystalline toxin of molecular weight 158,000. The specific toxicity of the component obtained by Wagman was 30% less than that of the starting material (7). In the present study, at low protein concentration and approximately physiological pH, the toxic fraction had a molecular weight of 150,000 and a Stokes radius of 48 A. Since many biologically active proteins consist of sub-

units (29), it is possible that the α protein is not a single polypeptide chain.

Although the β fraction produced a single band in Ouchterlony double diffusion tests with rabbit antiserum against crystalline toxin, or antiserum against chromatographically isolated β component (24), it appeared to contain more than one component when analyzed at pH 8.0 on DEAE-cellulose columns. Crestfield, Stein, and Moore (30) found that the aggregated forms of bovine pancreatic ribonuclease could be separated from monomers on ion exchange columns. Thus, the β subfractions were considered as the aggregated hemagglutinin in species of different molecular weights. The validity of this assumption was supported by ultracentrifuge experiments (24) in which, under all conditions of pH and ionic strength used, no more than four components were observed. Of these, one was the toxic α component with $s_{20,w}$ = 7.2, and the other three components were of $s_{20,w}$ = 13, 16, and 23, which correspond to the molecular weights of the β_1, β_2, and β_3 components, respectively. The molecular weights of these three components, estimated by gel filtration, were 290,000, 500,000, and 900,000, respectively. From these molecular weights and from their common biological activities, these hemagglutinin components seemed to be one entity in different states of aggregation. Furthermore, it appeared that the β_2 fraction was a common component of the three fractions because of its invariable appearance upon rechromatography of each of these fractions. The hemagglutinins of the crystalline toxin may have dissociated on the column into distinct fractions under the influence of increasing ionic strength during elution. The amount of material eluted with a stepwise gradient as the β_2 fraction was consistently larger than that obtained with a linear gradient. One possible interpretation is that, after the formation of β_1 and β_3 species under a linear gradient, only a small amount of hemagglutinin remained on the column in the form of β_2, which eluted much later. In the stepwise gradient elution, the formation of β_2 was suddenly interrupted by the application of 0.5 M Cl^-, and as a consequence the hemagglutinin remaining on the column eluted as β_2.

Our success in separating the hemagglutinin from the toxic moiety of the crystalline toxin is in agreement with the observations of others, who showed that the hemagglutinin could be removed from the toxin by adsorption on red blood cells (4). Wagman (5) had separated in the ultracentrifuge a hemagglutinin of $s_{20,w}$ = 14 from a toxic component of $s_{20,w}$ = 7. These authors overlooked the possibility that the neurotoxin and hemagglutinin might be different proteins rather than the same proteins in various forms of aggregation. Wagman (7) found that the $s_{20,w}$ = 7 fraction had a lower tyrosine content than the crystalline toxin, and that its absorption spectrum, between 250 and 300 mμ, differed distinctly from that of the crystalline toxin. We have also found this difference in absorption spectra between the α and β fractions and the crystalline toxin[1]; this suggests the presence of two different proteins. Furthermore, the α fraction differed from the β fraction in immunoelectrophoretic mobility (12), in Ouchterlony gel double diffusion tests (24), in the ultracentrifuge (24), and in gel filtration analysis. Our preliminary amino acid analysis of the α and β fractions and of the crystalline toxin also showed that the α fraction is significantly different from both the β fraction and the crystalline toxin.

Heckly, Hildebrand, and Lamanna (31) found that the toxin in lymph which drains the intestinal wall of orally intoxicated animals had a mean $s_{20,w}$ value of 7.9. Zacks *et al.* (32), who studied the site of deposition of *C. botulinum* type B toxin labeled with ferritin granules by electron microscopy, demonstrated the presence of these granules in the primary and secondary clefts of the myoneural junctions of animals that had received injections. A large proportion of these granules were 100 to 150 A apart. Since this spacing was observed only when ferritin-labeled toxin was used for injections, not ferritin alone, they proposed that the intervening space between ferritin granules was occupied by the toxin molecule.

It is recognized that the Stokes radius of a protein may not exactly express its actual dimension. However, if it is assumed that the molecular dimension of the α component obtained by physicochemical means ($s_{20,w}$ = 7.2 and 96 A diameter) is close to its actual dimensions, these values agree well with the corresponding values for the *C. botulinum* toxin found in body fluids and at myoneural junctions. Therefore, it is perhaps not too hazardous to speculate that the α fraction of type A toxin, with a diameter of 96 A and $s_{20,w}$ = 7.2, can, without further dissociation, penetrate the intestinal wall and reach the receptor sites at the myoneural junctions.

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[1] B. R. DasGupta and D. A. Boroff, unpublished observations.

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